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(72) Inventor: Baker, Kenneth Matthew
Clos de Bergoje 21
B-1160 Brussels (BE)

Baudichau, Alain Edmond
Rue Ryndick 2
B-5991 Tourinnes-la-Grosse (BE)

Lardinois, Pierre Fernand
Rue de la Citronnelle 21A
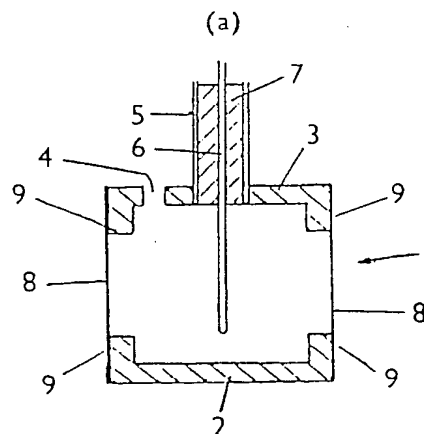B-1348 Louvain-la-Neuve (BE)

Rouxhet, Paul Gérard Unité C.I.F.A.
Unité de Chimie de Surface Place Croix du Sud No.1
B-1348 Louvain-la-Neuve (BE)

Stone, William Emile Ernest Unité C.I.F.A.
Unité de Chimie de Surface Place Croix du Sud No.1
B-1348 Louvain-la-Neuve (BE)

(74) Representative: Lunt, John Cooper et al
Monsanto Europe S.A. Patent Department Avenue de
Tervuren 270-272 Letter Box No 1
B-1150 Brussels (BE)

(54) Immobilised enzymes and their use in aminoacid electrosensors.

(57) The enzymes L-methionine gamma lyase, L-lysine decarboxylase, L-aspartase and L-tryptophanase are immobilised by contacting the enzyme with a latex of polymer particles having a negative surface charge such that the electrophoretic mobility of the latex has a negative value of $-2.0 \ 10^{-8}m^2V^{-1}sec^{-1}$ or below when measured at pH 7 in 0.01 M KNO_3 and a polymer solids concentration of 30 mg/l, the polymer particles also having a hydrophobic surface such that the contact angle of a 1 microlitre droplet of distilled water with a plane horizontal surface formed by drying and compacting the latex particles is 70° or more. The polymer-immobilised enzymes can be used for assaying the corresponding aminoacids in aqueous solutions using an electrosensor comprising a pH-sensitive electrode (6) located in a sensing zone or chamber (1) containing the enzyme/polymer particles as an aqueous dispersion or in water-dispersible form.

(a)

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Description

Immobilised Enzymes and their use in Aminoacid Electrosensors

This invention relates to the immobilisation of enzymes, more particularly to the immobilisation of enzymes such as L-methionine gamma-lyase and L-lysine decarboxylase. The invention also relates to an electrosensor for assaying aminoacids in solution, comprising a pH-responsive electrode in combination with an immobilised enzyme which degrades the aminoacid with an accompanying change in the pH of the solution.

Immobilised enzymes and their rôle in biocatalytic reactions are of considerable technological importance. Carriers which have been used to immobilise enzymes include polymer latices. In most instances, the polymers concerned have been derived from monomers or comonomers containing functional groups. For instance US-A-4 064 080 discloses latices of styrene polymers having terminal aminophenylthio groups for immobilising proteins; Bahadur et al, Makromol. Chem. (1985), 186, 1387 describe core-shell latices of poly(methyl methacrylate-co-acrylic acid) having carboxyl groups on the surface of the polymer particles, and the immobilisation of alpha-chymotrypsin on the surface of the latex particles by chemical bonding using a carbodiimide coupling agent; and Hoshino et al, Kobunshi Ronbunshu, (1985), 42 (5), 305 describe the use of hydrolysed styrene-N-hydroxymethyl acrylamide copolymer latices to immobilise alpha-amylase.

The use of pH sensors to monitor enzyme-catalysed decompositions has also been described in the literature. For example Ianniello and Yacynych, Anal. Chim. Acta (1983), 146, 249 report the use of an iridium dioxide-coated metal, for example titanium, as a pH responsive electrode for monitoring the decomposition of urea by urease. The urease was immobilised on to the iridium oxide by physical entrapment in a poly(vinyl chloride) film or by a covalent attachment via a cyanuric chloride linkage.

Fung et al, Analytical Chemistry, (1979) 51, 2319 describe a potentiometric enzyme electrode for the assay of methionine in solution. It was prepared by coating a layer of methionine lyase immobilised in bovine serum albumin cross-linked with glutaraldehyde on to an ammonia gas sensing electrode.

The non-covalent bonding of enzymes to a support surface is often a reversible process, and the equilibrium concentration of adsorbed enzyme may not be a useful amount. Moreover, enzyme which is adsorbed is frequently at least partially deactivated by distortion of its structure by bonding forces. The problem of deactivation by distortion can be particularly acute where regions of the enzyme are covalently bonded directly or through a bonding agent to functional groups on the surface of the support. This means that the desirable objective of immobilising an enzyme on a support without substantial loss of activity can only be met where there is a high degree of specificity between the enzyme, the support and the way in which they are linked.

In previously described biosensor devices comprising a sensor electrode in combination with an immobilised enzyme, the enzyme has been present as a dispersion in a relatively rigid film of polymeric material on the surface of the electrode or as a covalently-bonded coating on the surface of the electrode. Immobilisation in a rigid film imposes limitations on the accessibility of the substrate to the enzyme and on the reproducibility of such devices. The use of covalent bonding to immobilise the enzymes on the electrode surface restricts the amount of enzyme available in the system, while both methods of immobilisation limit the speed of response of the device.

We have found that the enzymes L-methionine gamma-lyase and L-lysine decarboxylase can be immobilised, with high enzyme activity retention, on polymer particles having defined characteristics. The same polymer particles can be used, somewhat less effectively, for the immobilisation of L-aspartase and of L-tryptophanase. Such particles carrying the immobilised enzyme can be used in conjunction with a pH-responsive electrode for the assay of the corresponding aminoacids in solution.

Accordingly, the invention provides a method for the immobilisation of an enzyme selected from L-methionine gamma lyase, L-lysine decarboxylase, L-aspartase and L-tryptophanase, which comprises contacting the enzyme with a latex of polymer particles having a negative surface charge such that the electrophoretic mobility of the latex has a negative value of $-2.0 \ 10^{-8} m^2 V^{-1} sec^{-1}$ or below (i.e. more negative) when measured at pH 7 in 0.01 M KNO_3 and a polymer solids concentration of 30 mg/l, the polymer particles also having a hydrophobic surface such that the contact angle of a 1 microlitre droplet of distilled water with a plane horizontal surface formed by drying and compacting the latex particles is 70° or more.

The invention further includes an electrosensor for use in assaying an aminoacid selected from L-methionine, L-lysine, L-aspartic acid and L-tryptophan, said electrosensor comprising a pH-responsive electrode located in a sensing zone containing polymer particles which have had the correspondingly selected enzyme immobilised thereon by the method of the invention, the said particles being present as an aqueous dispersion or in water-dispersible form.

The electrosensor of the invention has the advantage over previously described biosensors for enzymes that although the enzyme is immobilised on the surface of the polymer particles, the particles themselves are mobile or potentially mobile within the sensing zone. Access of the substrate to the enzyme is thereby vastly improved in comparison with previous systems in which the enzyme carrier is itself immobile.

Latices which can be used in the method of the invention can, for example, have an average particle size in the range 0.1 to 5 micrometres. Preferred latices have particles with an average size in the range 0.3 to 3 micrometres, more especially 0.6 to 1 micrometre. For certain applications, including use in the electrosensor of the invention, particles having a relatively narrow size distribution are preferred. Particles in latices produced by the emulsion polymerisation of monomers typically meet this last requirement.

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Many polymers are produced by emulsion or suspension polymerisation of a monomer or monomers using persulphate as a component of the polymerisation catalyst. This results in the presence of a small number of residual ionic groups derived from the persulphate catalyst, for example $-O-SO_3^-$, on the surface of the latex particles. In the absence of cationic surface groups, such groups confer a negative surface charge on the particles. Where the monomer or monomers from which the polymer is derived is non-polar, or substantially so, the residual anionic groups following a persulphate-catalysed polymerisation are normally sufficient to provide a latex having an electrophoretic mobility within the range required in the method of the invention. Good results have been obtained using latices of polystyrene and of a copolymer of styrene with a small amount of carboxylic monomer, for instance acrylic or methacrylic acid. As is well known, however, styrene will copolymerise in various proportions with a wide range of comonomers. It is believed that any of the considerable number of such copolymer latices could be used to immobilise enzymes in accordance with the method of the invention provided that the copolymer particles have a negative surface charge and a hydrophobicity as defined above.

It is also possible to modify the surface of the latex particles by mixing the latex with solutions of ionic or polar substances and then recovering and redispersing the modified latex particles. For example solutions of metallic salts, especially salts of multivalent metals such as aluminium, iron, titanium or zinc, or polar and ionic polymers such as chitosan, can be used for this purpose. The amounts of such modifiers used and the conditions of modification should be such that the electrophoretic mobility and hydrophobicity of the modified latex are within the limits specified above.

Preferably, latices for use in the invention have electrophoretic mobilities of from about -3.5 to about -6.5, for example from about -4.5 to about -5.5, $10^{-8}m^2V^{-1}sec^{-1}$ when measured at pH 7 in 0.01 M KNO_3 and a polymer solids concentration of 30 mg/l, and hydrophobic surfaces such that the aforesaid contact angle is from 70 to 150°, for example from 100 to 130°.

In the electrosensor of the invention, unlike the prior art electrosensors, the immobilised enzyme is not present in a rigid film coated on to the surface of the electrode. It is however confined to a sensing zone in which the pH-responsive electrode is located. In one embodiment, the electrosensor comprises a half cell adapted for use in conjunction with an exterior reference electrode. The sensing zone is within a container constructed of materials such that when the container contains and is surrounded by a solution containing the aminoacid to be assayed, latex particles with adsorbed enzyme are retained within the container, but the aminoacid can diffuse into and out of the container.

In another embodiment, the electrosensor comprises a container containing latex particles with adsorbed enzyme, and is adapted to accommodate both a pH-sensitive electrode and a reference electrode.

The invention further includes a method of assaying an aminoacid, which comprises introducing a solution containing the aminoacid into the sensing zone of an electrosensor of the invention, said solution being in contact with a reference electrode, and said sensing zone also containing pyridoxal-5-phosphate as a co-enzyme, measuring the change in the potential difference between the pH-responsive electrode and the reference electrode over a fixed time interval and correlating the said change with the amount of aminoacid in the solution.

In the drawings, Fig (1) a shows a cross-section through an electrolytic half-cell which can be used to determine an aminoacid in accordance with the invention. Figs 1(b) and 1(c) show cross-sections of electrolytic cells useful for the same purpose. Figs 2-6 illustrate graphically various results obtained in the Examples set out below.

The half-cell of Fig 1(a) comprises a generally cubic chamber (1), of which the lower horizontal face (2) is continuous. Size is not critical, but the half-cell used in the experiments described in the Examples below had an external dimension of approximately 2.5 cm. The upper horizontal face (3) has an opening (4) to provide access to the interior of the chamber, and is attached centrally to a cylindrical sleeve (5) within which a pH-sensitive wire electrode (6) is held coaxially by means of packing (7). The vertical faces of the cube have square cut-out areas which are covered with semi-permeable membranes (8) attached to the edges (9) of the vertical faces of the cube. The dimensions of the cubic chamber are not critical; that used in the Examples described below had an outside dimension of approximately 27 mm and a wall thickness of approximately 2 mm. All materials of construction except the wire electrode are electrically non-conductive. Various polymeric materials, e.g. polypropylene, poly(tetra-fluoroethylene), polycarbonates, acrylonitrilestyrene-butadiene polymer blends or polyamides can be used. Rislan®, which is a blend of nylon 11 and nylon 12, has been found to be particularly suitable. pH-sensitive wire electrodes comprise a metal oxide coating on a metal wire, and various combinations have been described in the literature. In the half-cell used in the Examples below, the pH-sensitive wire electrode consisted of a titanium wire coated with iridium oxide with a further external coating of a fluoro-sulphonyl copolymer (see US-A-4 536 274). The semi-permeable membranes are required to retain latex particles but to be permeable to aminoacids. Membranes of cellulose acetate and of a polycarbonate film having a pore size of 0.45 micro-metres, each having a thickness of about 10 micrometres, have been found to be suitable.

In Figs 1(b) and 1(c), each cell comprises a container (10) with a lid (11), both made from a plastics material such as polypropylene or polystyrene. The container is conveniently cylindrical in shape, with a typical external diameter of approximately 10 mm and a typical height of 7 mm. In the cell of Fig 1(b), a pH-sensitive wire electrode (12) and a reference electrode (13), typically a silver chloride-coated silver wire, are fixed as inserts through the lid (11). A connector (14) has terminals (15) which are adapted to make a push fit over the upper

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ends of the electrodes (12) and (13) exterior to the lid of the container, and has conductive means (16) to connect the terminals (15) with a pH meter. The lid can be made to be removable, or alternatively, it can be of a thickness which can be penetrated by a hollow needle so that liquids can be injected into the container from a syringe.

In the cell of Fig 1(c), the lid (11) has two openings (17) which permit the insertion of a pH-sensitive wire electrode (18) and a reference electrode (19), the electrodes being inset at their upper ends into a disc or bar (20) of electrically insulating material which forms part of a connector (21). When the cell is in use, the disc or bar is adapted to rest on the lid of the container. The connector (21) is also provided with conductive means (22) to connect the electrodes with a pH meter.

In the Examples described below, two latices, Estapor© latex K109 (A) and Estapor© latex PSI 480 (B), both manufactured by Rhône- Poulenc, were used. Each is described by the manufacturers as a polystyrene latex containing 10% by weight of solids in suspension and a particle diameter of approximately 0.80 micrometres, with latex B additionally having carboxyl surface functional groups.

Various characteristics of the latices were investigated using X-ray photoelectron spectroscopy (XPS), infra-red spectroscopy (IRS), microelectrophoresis, and contact angle measurements. To obtain samples for XPS, IRS and contact angle measurements, the original latices were washed by centrifugation and resuspension twice in distilled water. The final sediment was freeze dried.

For XPS, the powder was pressed with a spatula in stainless steel troughs (8 mm in diameter), the surface being smoothed with the spatula. For contact angle measurements, ten wells (4 mm in diameter, 0.3 mm deep) hollowed in a polyacetal plate were filled in the same way. For recording IR spectra, thick paper with a hole of 5 mm diameter was used as the sample holder; the latex powder was placed in the hole and pressed under a pressure of about 30 MPa.

XPS spectra were recorded with a Vacuum Generator ESCA 3 spectrometer interfaced with a Tracor Northern Signal accumulator using a non-monochromatized Mg K source (14kV, 20 mA). The analysis energy was 50 eV and the electron take-off angle 45°.

All functional groups were assumed to be localised at the surface of the latex particles, and quantitative surface analysis was carried out using the integrated surface of the peaks and the empirical sensitivity factors of Wagner et al. (Surf. Interface Anal., (1981), $\underline{3}$(5), 211-225) and Wagner, (J. Electron Spectrosc. Relat. Phenom., (1983), $\underline{32}$, 99-102).

The XPS spectra of all latices were dominated by the peak of C_{1s} electrons, the binding energy (BE) of which was set at 285.0 eV and served as reference for the energy scale. The spectra showed an S_{2p} peak, with a BE between 168.7 eV and 169.7 eV, which is typical of groups ($-OSO_3^-$). The presence of such groups on the surface of all latices was attributed to the initiation of polymerization by $S_2O_8^=$ which produces SO_4^- radicals a $-O-SO_3^-$ group remains attached at each end of the polymer chain. An O_{1s} peak, between 532.5 eV and 533.5 eV, contained a contribution of oxygen from $-O-SO_3^-$ groups and from carboxyl groups.

The mean values of atomic concentration ratios (C_x/C_c) (x = oxygen or sulphur) in latex samples and calculated values for the superficial concentrations of oxygen and sulphur atoms, (O) and (S) are given in Table 1 below.

Table 1

Latex	C_s/C_c	C_o/C_c	$(S)nm^{-2}$	$(O)nm^{-2}$
A-sample 1	0.0013	0.011	0.10	0.46
A-sample 2	0.0011	0.015	0.09	0.62
B-sample 1	0.0020	0.018	0.16	0.75
B-sample 2	0.0015	0.019	0.12	0.79

Infrared absorption spectra (2000 to 1500 cm^{-1}) were recorded on a Perkin Elmer 580 B spectrophotometer equiped with a Data Station microprocessor. The intensity of the whole spectrum was normalised by setting the absorbance at 1945 cm^{-1} at a given value, thus eliminating the effect of variations in pellet thickness. The contribution of the carboxyl groups at 1750-1700 cm^{-1} was determined by subtracting the spectrum of a polystyrene latex containing no carboxyl. Calculated surface concentrations of carboxyl groups (groups nm^{-2}) were: Latex A, sample 1, 0.17; Latex B, sample 1, 0.33; Latex B, sample 2, 0.4.

The microelectrophoretic mobilities of the latices were measured with a Lazer Zee-Meter 500 (Pen-Kem) in a rectangular polymethylmethacrylate cell. The total volume of suspension introduced was about 25 ml. The applied voltage was 30 to 50 V.

Electrophoretic mobilities were determined at various pH values in both 0.01 M and 0.001 M KNO_3. They were also determined in the L-methionine gamma lyase medium diluted 10 times (0.01 M in phosphate) (MGL) and in the lysine decarboxylase medium diluted twice (0.01 M in phosphate) (LD). Both latices were negatively charged from pH 3 to pH 10.

Values for electrophoretic mobility (with an uncertainty factor of ± 0.5 unit of mobility[1] at pH 7.0 ± 0.2 are given in Table 2 below.

: Unit = $10^{-8} m^2 V^{-1} sec^{-1}$

Table 2

Medium Latex	KNO₃ 0.001 M pH 7	KNO₃ 0.01 M pH 7	MGL pH 7.2	LD pH 6.8	
A-sample 1	-5.0	-4.5	-5.0	-3.0	*5*
B-sample 1	-5.0	-5.0	-5.4	-	

For contact angle measurements, small drops (1 microlitre) of distilled water were deposited on the flat, horizontal surface of the samples. The height (h) and width (w) of the base of the drop were measured on a *10* magnified picture projected on a screen. The contact angle (φ) between the solid surface and the tangent to the drop at the solid-liquid-gas contact point, is calculated by means of the equation

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$$\emptyset = 2 \arctan \frac{2h}{w}$$

The higher the value of φ, the more hydrophobic is the surface. The results weRNA lare: Latex A, sample 1, *20* 122° ± 7°; Latex B; sample 1, 119° ± 4°.
The invention is illustrated by the following Examples:

Example 1 *25*

This Example describes the immobilisation of L-methionine gamma lyase on polymer particles and the use of the immobilised enzyme in a biosensor to determine L-methionine.
L-methionine gamma-lyase solution was provided by Dr. K. Soda, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan. It has a molecular weight of about 172.000 and consists of four subunits *30* with identical molecular weights. It contains 4 mol of pyridoxal 5'-phosphate per mole of enzyme (Soda et al, Anal. Biochem, 1984, 138, 421-4).
The medium of the enzyme solution (Medium M) was 0.1 M potassium phosphate buffer at pH 7.2, containing 20 micro-M pyridoxal phosphate, 0.01% 2-mercaptoethanol, 1mM EDTA and 2% ethanol.
Enzymatic activity was determined by introducing into a tube 1 ml of a 0.2 M potassium phosphate buffer *35* solution, 500 microlitres of 0.1 M L-methionine, 100 microlitres of 0.1 mM pyridoxal phosphate and 50 microlitres of enzyme solution, followed by dilution with water to a total volume of 2 ml.
The tube was incubated at 37°C for 10 minutes and the reaction then terminated by the addition of 0.25 ml of 50% tri-chloroacetic acid. After centrifugation, alpha-ketobutyrate in the supernatant was determined using 3-methyl-2-benzothiazoline hydrazone as described by Soda, Analytical Biochemistry (1968), 25, 228-235. One *40* unit of enzyme activity (U) corresponds to the production of 1 micromole of alpha-ketobutyrate in 1 minute.
Latices were washed three times with Medium M referred to above by centrifuging and resuspension to give finally a latex containing 10 mg polymer/ml.
Adsorption of the enzyme by the latex was measured by mixing 0.1 ml each of the latex and the enzyme solution and making up to a volume of 1 ml by the addition of Medium M in a tube having a volume of 3.5 ml. The *45* mixture was left for 1 hour at ambient temperature and then centrifuged at 10⁴ rpm for 15 minutes to give a compacted pellet of latex particles in the base of the tube and a supernatant solution. The activity of the adsorbed enzyme was measured by washing the pellet once with Medium M, redispersing the pellet in Medium M to a volume of 0.5 ml, and then determining enzyme activity in the suspension by the method described above for the original enzyme solution. *50*
Results obtained are set out in the table below. The first line gives a summary of results with 18 samples of the latex, each of 3.75 mg. The second line is a summary of results with 2 samples each of 1.75 mg. The third, fourth and fifth lines show the results with one sample each of 1.5 mg, 2.5 mg and 3.75 mg respectively.

Latex	mg. of carrier	Initial Activity in solution	Activity on pellet	Activity per mg. of carrier	
A Sample 1	3.75	1.42 ± 0.2	1.33 ± 0.3	0.36 ± 0.06	*55*
	1.75	1.33 ± 0.03	0.725 ± 0.07	0.48 ± 0.06	
B Sample 1	1.50	1.8	0.9	0.6	*60*
	2.50	1.8	0.8	0.32	
	3.75	2.1	1.9	0.50	

The use of L-methionine gamma lyase immobilised on a latex in accordance with the invention in a biosensor *65*

for the determination of L-methionine, was investigated using the apparatus of Fig 1(a) described above.

To prepare immobilised enzyme for use in the determination of L-methionine, Latex A described above was washed three times with distilled water by centrifugation and redispersion, giving a final dispersion containing 10% by weight of polymer solids. Approximately 0.1 mg of enzyme was added to 700 mg of the final dispersion, and the mixture was allowed to stand at room temperature for 2 hours. It was then centrifuged and the supernatant liquid was decanted from the pellet of sediment.

The chamber of the half cell of Fig 1(a) was immersed in a 10^{-3} molar solution of phosphate buffer at a temperature of 25°C, together with a standard calomel electrode placed adjacently. The electrodes were connected to a pH meter, and the potential difference between the electrodes was noted. The procedure was repeated with a series of 10^{-3} molar phosphate buffer solutions containing 10^{-4} molar pyridoxal-5-phosphate and various concentrations of L-methionine. In other experiments, the effect of varying the pH of the buffer at a fixed (10^{-2}M) concentration of L-methionine was investigated. In each experiment in the series, a pellet of immobilised enzyme, prepared as described above, was introduced into the chamber of the half cell through the opening ((4) in Fig 1(a)) after immersion of the half cell in the solution. The potential difference between the electrodes before the introduction of the pellet was noted, and the potential difference was recorded at intervals after its introduction. The results obtained are shown in Figs 2, 3 and 4.

Fig 2 shows the direct plot of potential difference against time at a buffer pH of 7 and various concentrations of L-methionine. Fig 3 is a plot of the potential difference 30 minutes after the introduction of the enzyme against the concentration of L-methionine with a buffer pH of 7. Fig 4 is a plot of potential difference against time at the fixed concentration of L-methionine with buffer solutions at various pH values.

Example 2

This Example describes the use of L-lysine decarboxylase immobilised on polymer particles in a biosensor to determine L-lysine.

The enzyme used in these experiments was L-lysine decarboxylase Sigma type VIII available from Sigma Corporation, U.S.A.

A sample of latex A above was centrifuged and the sediment was redispersed in a phosphate buffer solution of pH 6. Centrifugation and redispersion were repeated twice.

5 ml of the final dispersion, containing 10% by weight of solids, was mixed with 4.4 mg of L-lysine decarboxylase and the mixture was allowed to stand at room temperature for 1 hour. It was then centrifuged, and the supernatant liquid was decanted from the pellet of sediment.
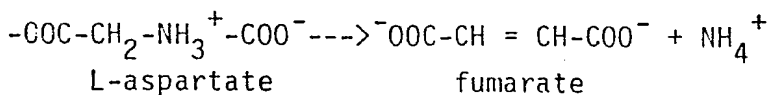
The chamber of the half-cell of Fig 1 (a) was immersed in a 10^{-2} molar solution of phosphate buffer at pH 5.92 and a temperature of 37°C, together with a standard calomel electrode placed adjacently. The electrodes were connected to a pH meter, and the potential difference between the electrodes was noted. The procedure was repeated with a series of 10^{-2} molar phosphate buffer solutions containing 10^{-4} molar pyridoxal-5-phosphate and various concentrations of L-lysine. In each experiment in the series, a pellet of immobilised enzyme, prepared as described above, was introduced into the chamber through the opening (4) in Fig 1 (a) after immersion of the half cell in the solution. The potential difference between the electrodes before the introduction of the pellet was noted and the potential difference was recorded for up to 20 minutes after its introduction. The results obtained are shown in Fig 5 and 6. Fig 5 shows direct plots of potential difference against time at the different L-lysine concentrations. Fig 6 is a plot of the value of the potential difference 5 minutes after the introduction of the enzyme against the concentration of L-lysine.

Example 3

This Example describes the immobilisation of L-aspartase on polymer particles.

L-aspartase extracted from Hafnia alvei (Bacterium cadaveris) was purchased from Sigma. L-aspartase has a molecular weight of 180000, an isoelectric point of 4.8 and optimum pH 7.8. The enzyme was dissolved in a phosphate buffer 0.1 M (KH_2PO_4 + K_2HPO_4) pH 7.2 and divided into portions which were frozen until required for use.

The enzymatic activity assay is based on the following reaction:

$$-COC-CH_2-NH_3^{+}-COO^{-} \longrightarrow {}^{-}OOC-CH = CH-COO^{-} + NH_4^{+}$$

L-aspartate   fumarate

and is measured by the fumarate production. One activity unit (U) converts 1.0 umole of of L-aspartate to fumarate per minute.

The measurement is carried out by introducing the following reagents into a quartz cell (1 cm light path).
- 1 ml-0.15 M Tris buffer. pH 8.5

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- 100 μl-0.6 M MgSO₄.7H₂O solution
- 100 μl-3mM EDTA disodium salt solution
- 300 μl-0.5 M L-aspartate substrate solution
- 1.4 ml-H₂O,

mixing by inversion, adding 100 μl enzyme solution, and diluting to obtain about 0.2 U or 100 μl of resuspended pellet. The contents of the cell are immediately mixed by inversion and the increase of absorbance (A*) at 240 nm versus time is recorded.

A linear increase in absorbance with time is observed. The units of enzymatic activity in the cell are given by the formula

$$\frac{\triangle A^* \times 3}{2.53 \times \triangle time}$$

where 2.53 is the extinction coefficient of potassium fumarate ($mM^{-1} . cm^{-1}$) and 3 is the final volume of the reaction mixture (ml).

Latex A was washed three times with 0.1 M phosphate buffer (pH 7.2) by centrifuging and resuspension. Adsorption of the enzyme by the latex was measured by adding a portion of enzyme solution having a predetermined activity to 3.75 ml of latex and making up the volume to 1 ml by the addition of buffer solution. The mixture was left for 1 hour at ambient temperature and then centrifuged at 10^4 rpm for 15 minutes to give a compacted pellet of polymer particles and a supernatant solution. The activity of the adsorbed enzyme was measured by washing the pellet once with the buffer, resuspending in a volume of 250 μl and then applying the method described above. In a second investigation, the above procedure was repeated using a phosphate buffer having a pH of 8.0.

Results obtained are set out below:

	Activity in initial solution (U)	Activity in pellet (U)
a) pH = 7.2	0.26	0.019
	0.30	0.014
	0.30	0.015
	0.23	0.015
b) pH = 8.0	0.30	0.015

The results indicate that a comparatively small fraction of the enzyme is adsorbed on the latex by this procedure. However, concurrent determinations of the residual enzymatic activity in the supernatant solution and of the total protein adsorbed suggested that proteins that are impurities in the commercial aspartase were adsorbed preferentially. Addition of a second portion of latex to the supernatant liquid would be expected to result in a higher value of the activity per mg carrier for this second portion.

Example 4

Experiments on the adsorption of L-trytophanase on latex A indicated that proteins that are impurities in the commercially-purchased enzyme are preferentially adsorbed relative to the enzyme itself when using washed but otherwise untreated latex. Pretreatment of the latex with aluminium nitrate improved the selectivity but without significantly increasing the amount of active enzyme adsorbed per unit of carrier. The treatment with aluminium nitrate had little effect on the electrophoretic mobility of the latex, but increased the hydrophobicity of the particles as shown by an increase in the angle of contact of a water droplet with a surface formed by drying and compacting the latex particles from about 125° to about 163°.

Claims

1. A method of immobilising an enzyme selected from L-methionine gamma lyase, L-lysine decarboxylase, L-aspartase and L-tryptophanase which comprises contacting the enzyme with a latex of polymer particles having a negative surface charge such that the electrophoretic mobility of the latex has a negative value of $-2.0 \ 10^{-8} m^2 V^{-1} sec^{-1}$ or below when measured at pH 7 in 0.01 M KNO_3 and a polymer solids concentration of 30 mg/l, the polymer particles also having a hydrophobic surface such that the contact angle of a 1 microlitre droplet of distilled water with a plane horizontal surface formed by drying

and compacting the latex particles is 70° or more.

2. A method according to Claim 1 in which the negative surface charge of the latex particles is such that the latex has an electrophoretic mobility of from about -3.5 to about -6.5 $10^{-8} m^2 V^{-1} sec^{-1}$ and the said contact angle is from 70 to 150°.

3. A method according to either of Claims 1 and 2 in which the average particle diameter of the latex is from about 0.6 to about 1.0 micrometres.

4. A method according to any of Claims 1 to 3 in which the polymer latex is a latex produced by the emulsion polymerisation of a monomer or of monomers using a persulphate polymerisation catalyst, and the negative surface charge is at least partly due to the presence of residual ionic groups derived from the persulphate.

5. A method according to Claim 4 in which the polymer is polystyrene or a copolymer of styrene with one or more comonomers having zero or negative polarity.

6. A method according to Claim 5 in which the polymer is a copolymer of styrene with a carboxylic monomer.

7. A method according to any of Claims 1 to 6 for the immobilisation of L-methionine gamma lyase or L-lysine decarboxylase, in which the negative surface charge of the latex particles is such that the latex has an electrophoretic mobility of from about -4.5 to about -5.5 10^{-8} $m^2 V^{-1} sec^{-1}$, and the hydrophobicity of the polymer particles is such that the said contact angle is from 100 to 130°.

8. An electrosensor for use in assaying an aminoacid selected from L-methionine, L-lysine, L-aspartic acid and L-tryptophan, said electrosensor comprising a pH-sensitive electrode located in a sensing zone containing polymer particles which have had a correspondingly selected enzyme immobilised thereon by the method of any of Claims 1 to 8, the said particles being present as an aqueous dispersion or in water-dispersible form.

9. An electrosensor according to Claim 8 in which the pH-sensitive electrode is a metal/metal oxide electrode.

10. An electrosensor according to Claim 9 in which the pH-sensitive electrode comprises iridium oxide on iridium or titanium, and the iridium oxide has a coating of a perfluorosulphonate polymer.

11. A method of assaying an aminoacid selected from L-methionine, L-lysine, L-aspartic acid and L-tryptophan, which comprises introducing an aqueous solution containing the aminoacid into the sensing zone of an electrosensor according to any of Claims 9 to 11 having the correspondingly selected enzyme immobilised on the polymer particles, said solution being in contact with a reference electrode and said sensing zone also containing pyridoxal 5-phosphate as a co-enzyme, measuring the change in the potential difference between the pH-responsive electrode and the reference electrode over a fixed time interval, and correlating the said change with the amount of aminoacid in the solution.
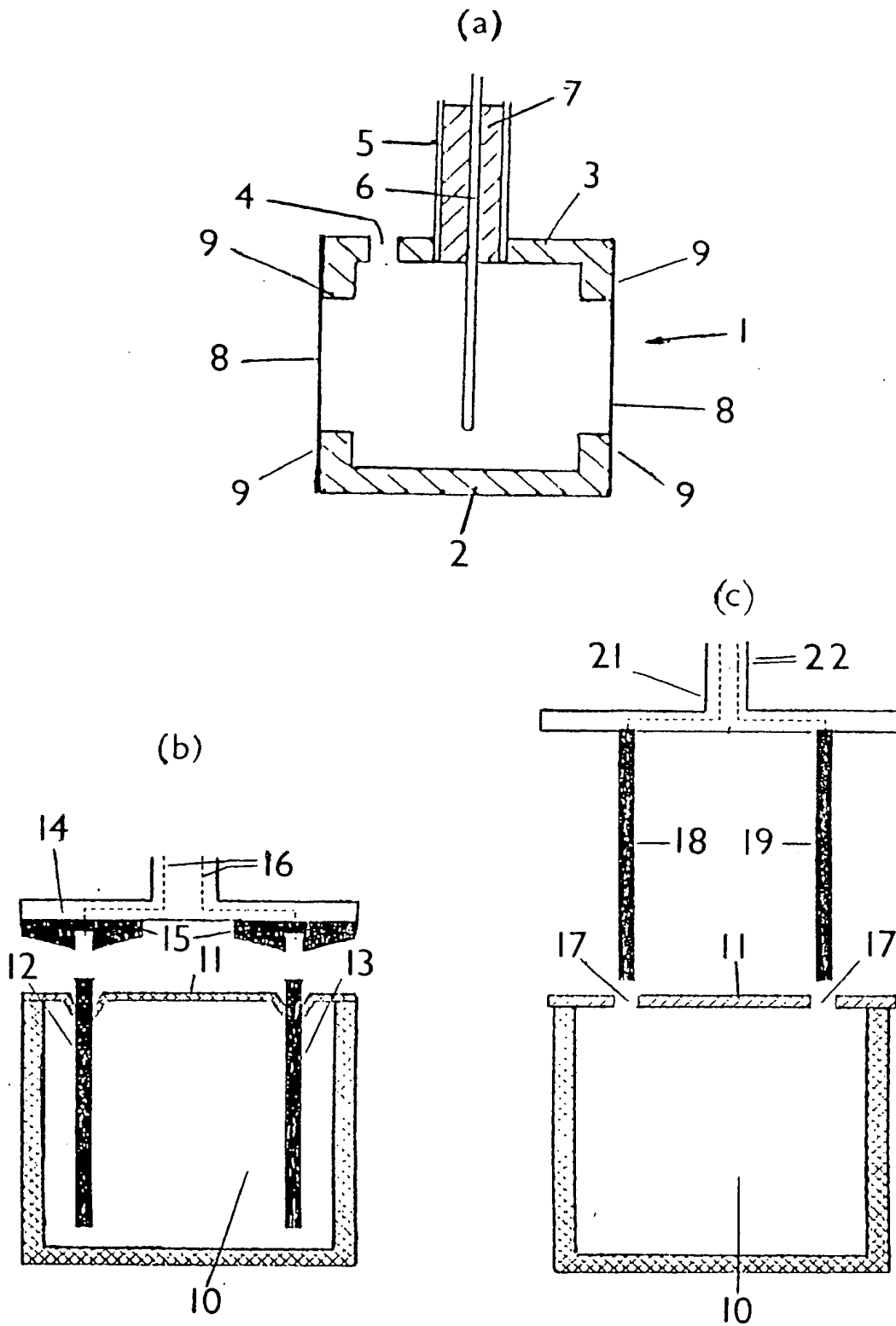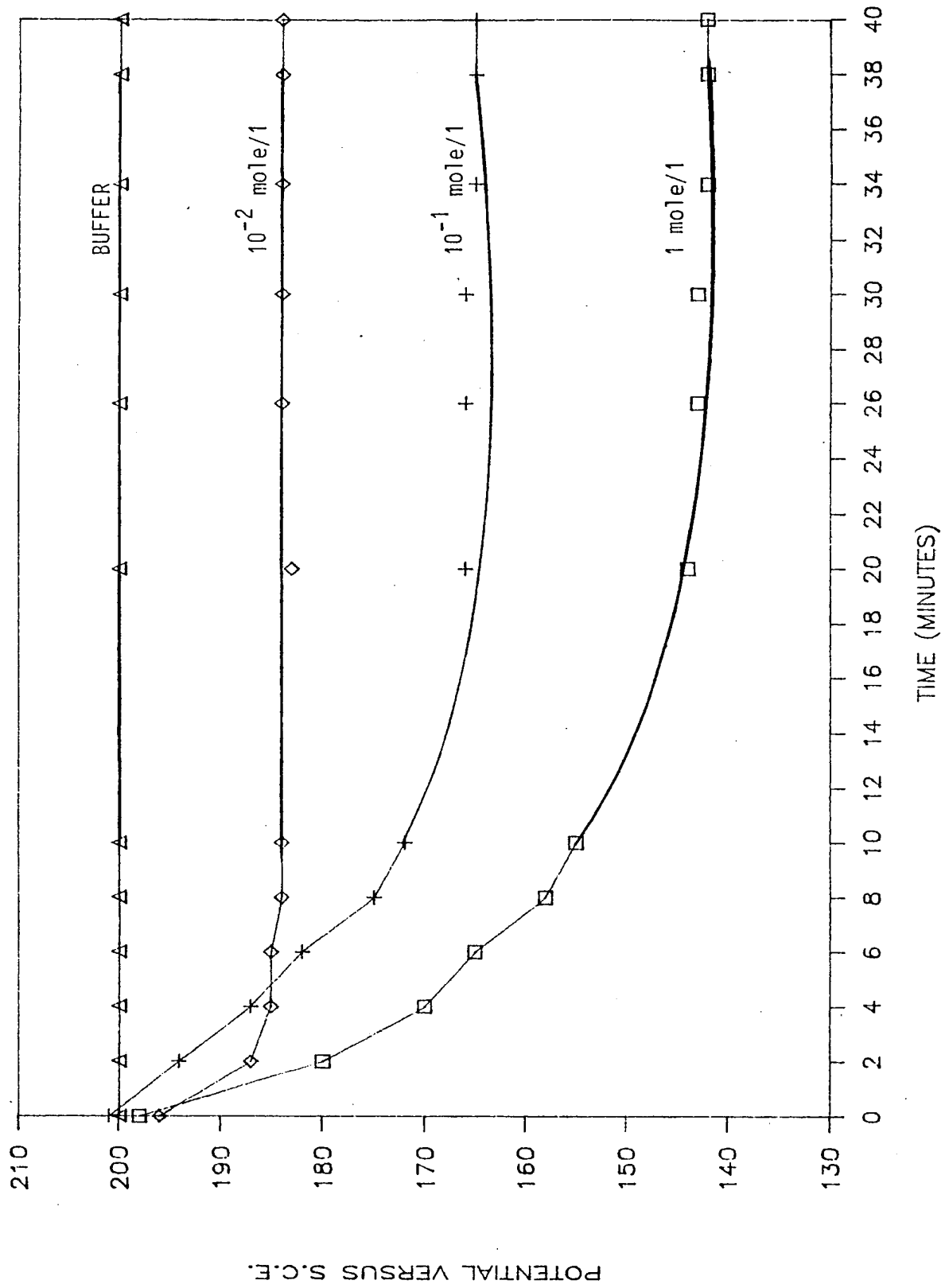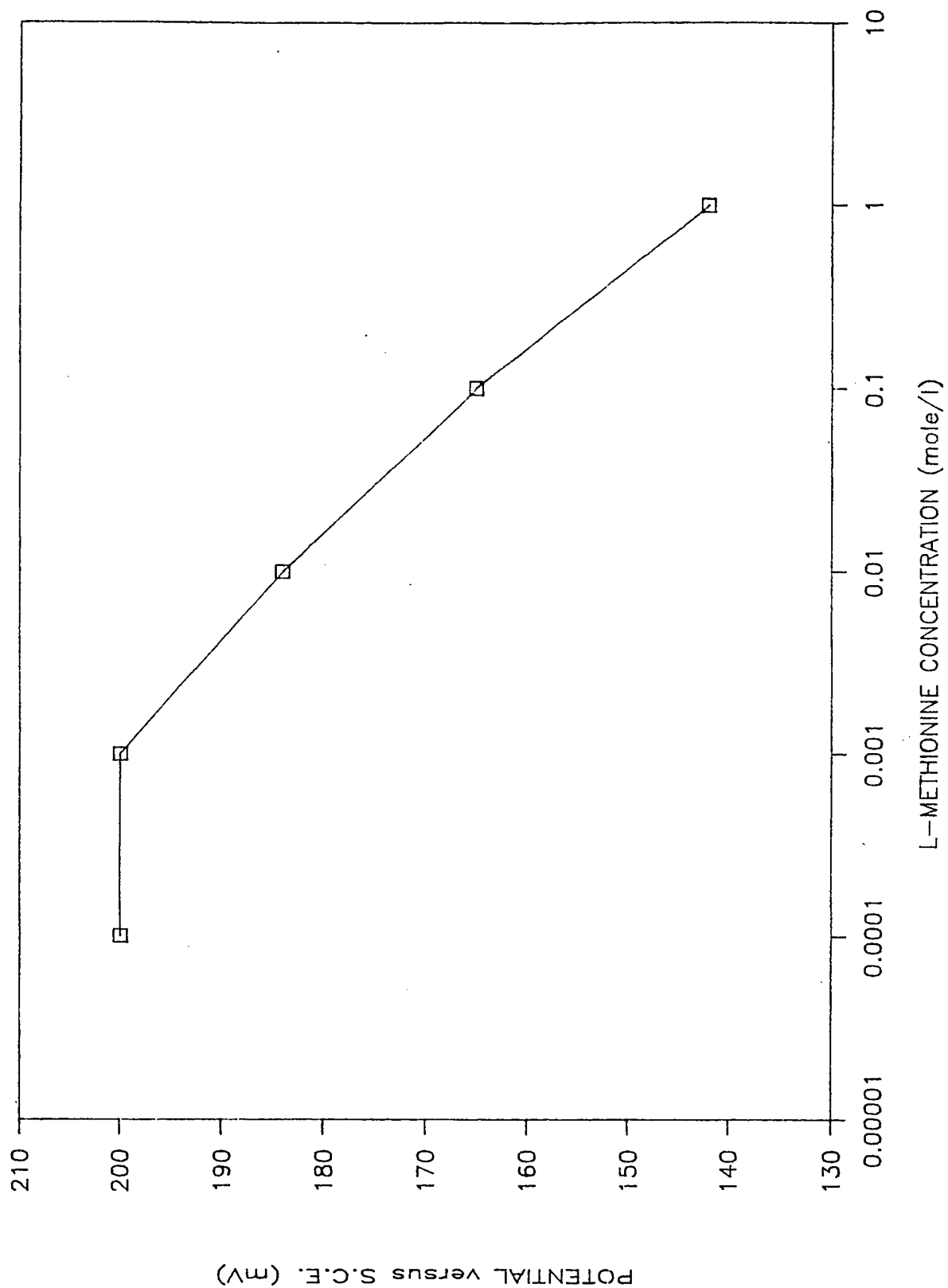
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FIGURE 1

(a)

(b)

(c)

FIGURE 2

FIGURE 3

L—METHIONINE CONCENTRATION (mole/l)

POTENTIAL versus S.C.E. (mV)

FIGURE 4

FIGURE 5

POTENTIAL VERSUS S.C.E.

TIME (minutes)

BUFFER

10^{-5} mole/l

10^{-4} mole/l

10^{-3} mole/l

10^{-2} mole/l

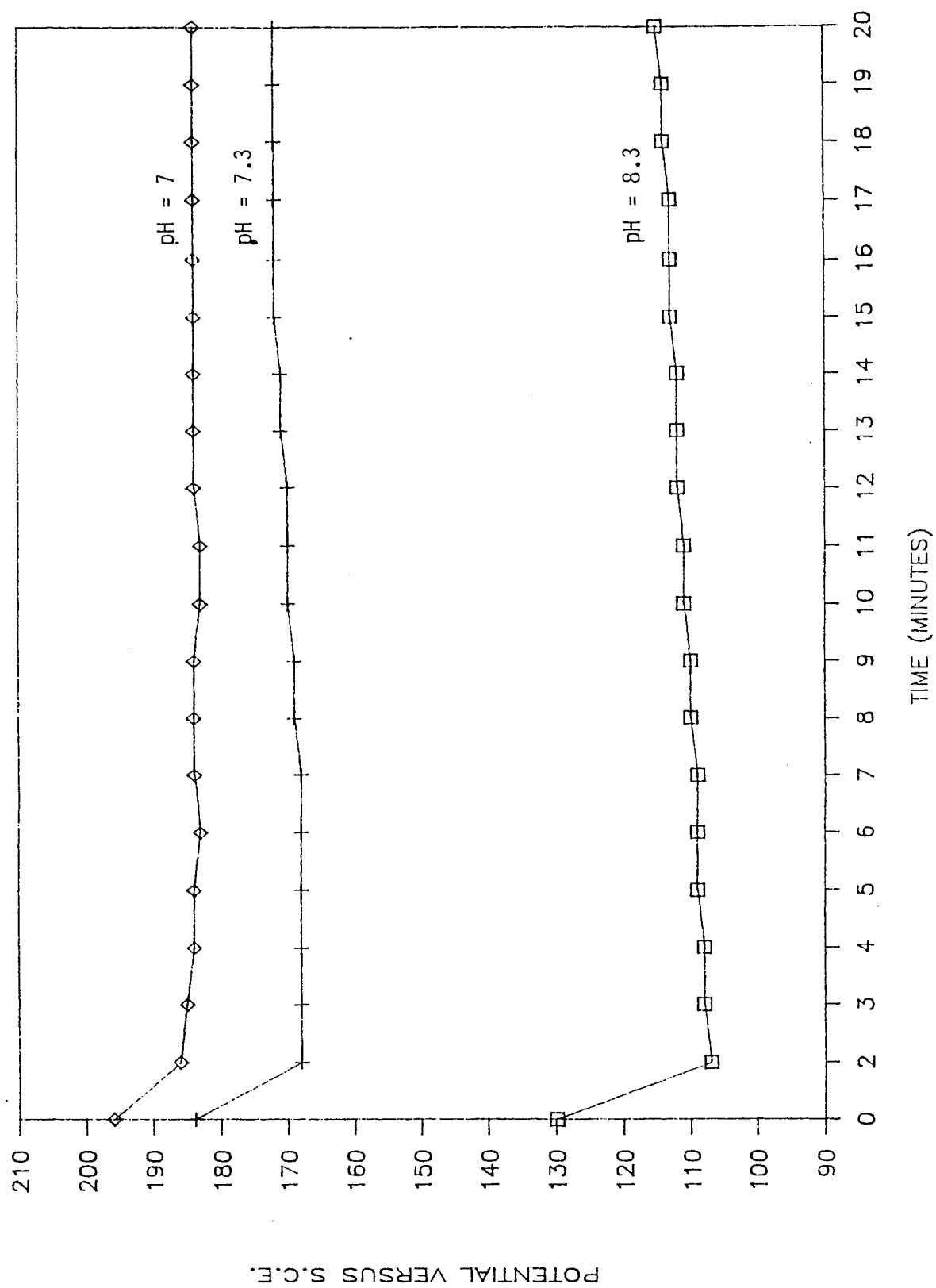FIGURE 6

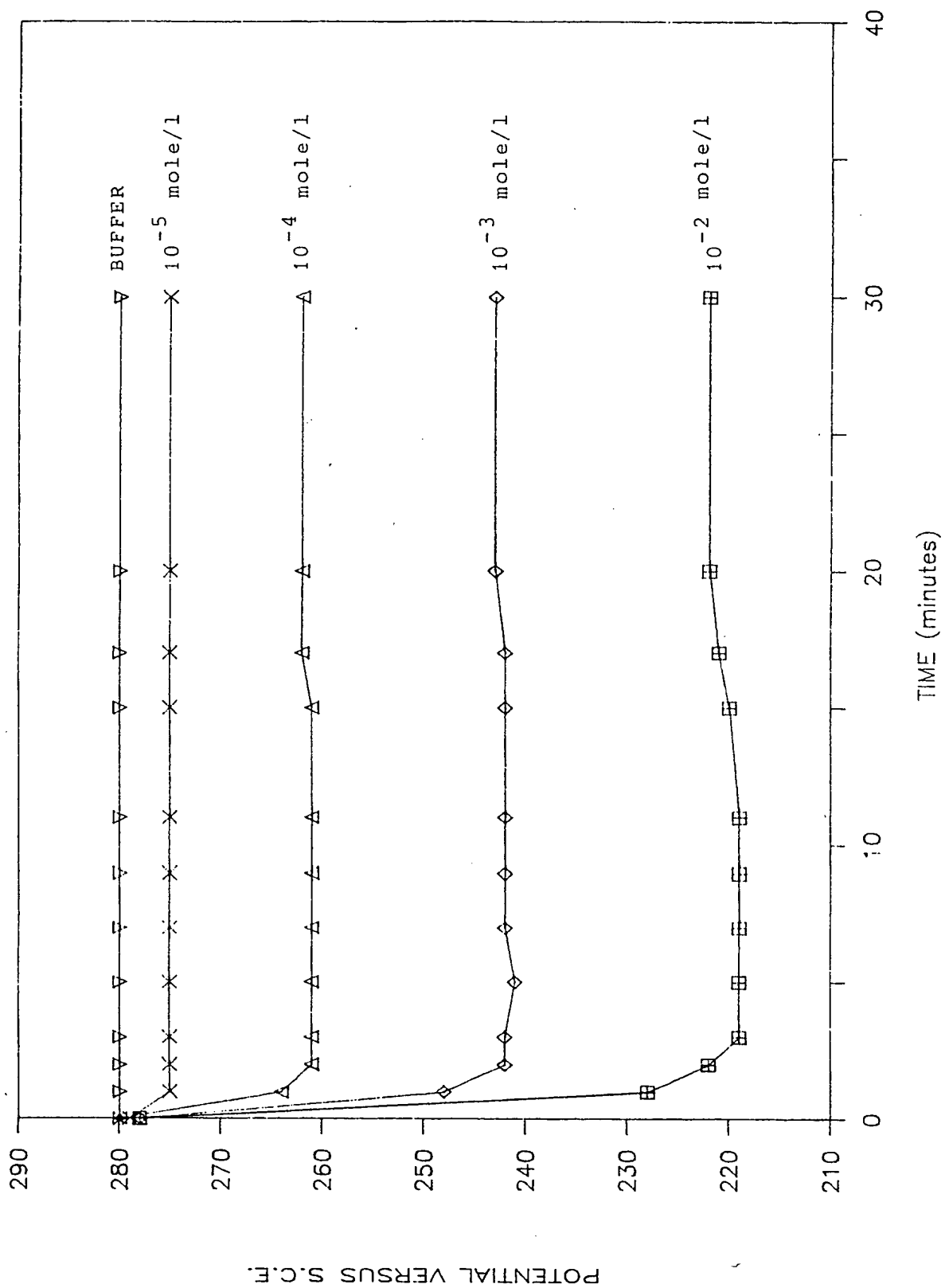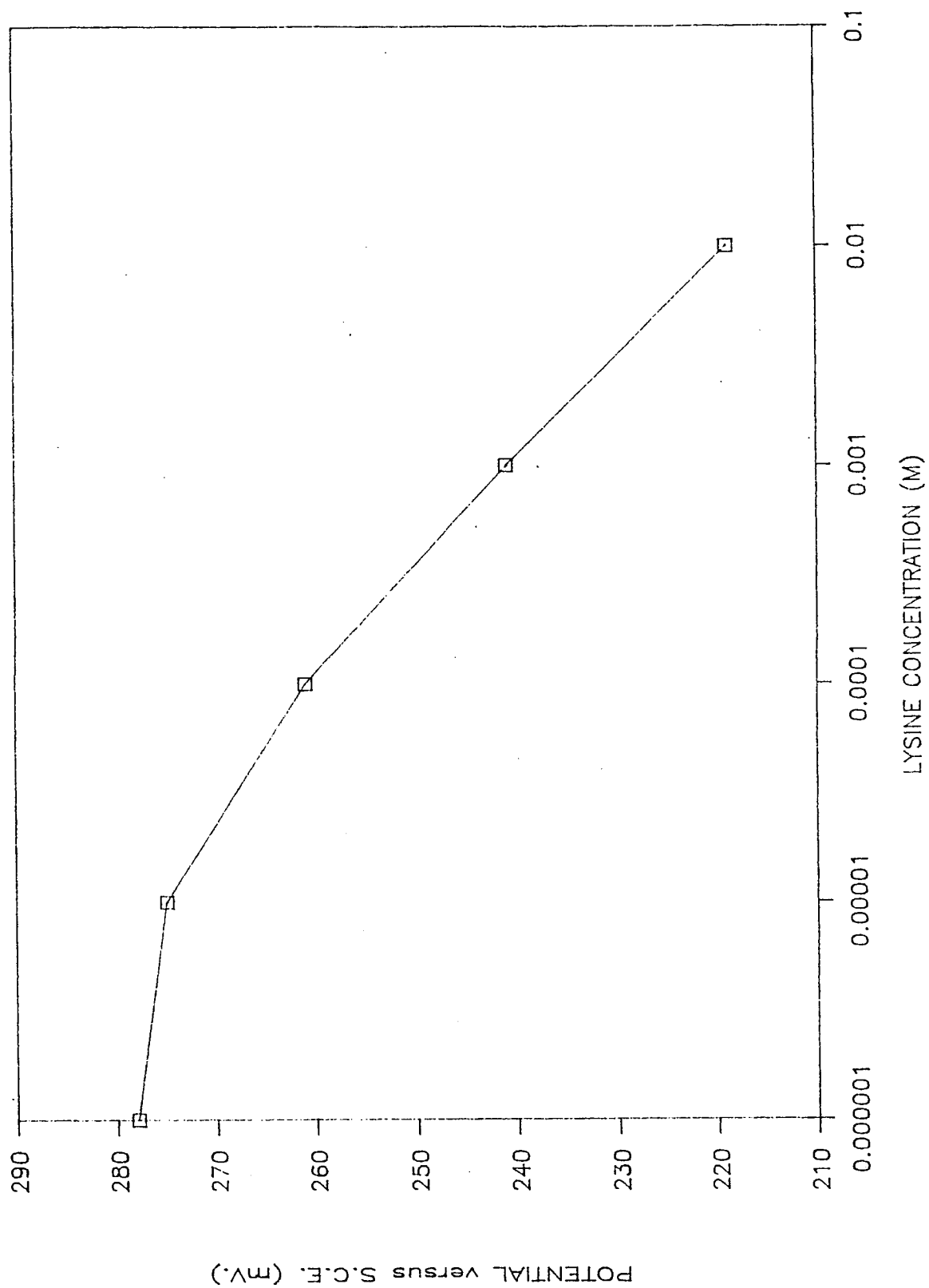

POTENTIAL versus S.C.E. (mV.)

LYSINE CONCENTRATION (M)

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP-A-0 132 998 (KYOWA HAKKO KOGYO CO., LTD) * Page 2, lines 18-31; page 3, lines 1-6 *	1-11	C 12 N 11/08 C 12 M 1/40 C 12 Q 1/00
A	EP-A-0 075 815 (BASF AG) * Pages 4-8 *	1-11	
A	US-A-4 195 129 (S. FUKUI) * Column 2, lines 23-32; column 6, lines 44-54; column 7, lines 9-13 *	1-11	
A	CHEMICAL ABSTRACTS, vol. 86, no. 17, 25th April 1977, page 190, abstract no. 116829g, Columbus, Ohio, US; & JP-A-76 125 790 (KYOWA HAKKO KOGYO CO., LTD) 02-11-1976 * Abstract *	1-11	
A	CHEMICAL ABSTRACTS, vol. 93, no. 17, 27th October 1980, page 495, abstract no. 166016e, Columbus, Ohio, US; A. TANAKA et al.: "Application of immobilized lysine decarboxylase tubes for automated analysis of L-lysine", & J. FERMENT. TECHNOL. 1980, 58(4), 391-4 * Abstract *	1-11	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 M
A,D	US-A-4 064 080 (J.-C. DANIEL) * Whole document *	1-11	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	13-02-1989	FERNANDEZ Y BRANAS F.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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